AD________________

AWARD NUMBER DAMD17-97-1-7310

TITLE: Breast Tumor Detection Utilizing Novel in situ Amplification Systems

PRINCIPAL INVESTIGATOR: Cassandra L. Smith, Ph.D.

CONTRACTING ORGANIZATION: Boston University
Boston, Massachusetts 02215

REPORT DATE: August 1999

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1999	Final (7 Jul 97 - 7 Jul 99)

4. TITLE AND SUBTITLE
Breast Tumor Detection Utilizing Novel in situ Amplification Systems

5. FUNDING NUMBERS
DAMD17-97-1-7310

6. AUTHOR(S)
Cassandra L. Smith, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Boston University
Boston, Massachusetts 02215

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

Although exisiting radiographic, ultrasound and MRI modalities for the the detection of breast canter are useful, they are less than ideal. Nuclear medicine approaches using raiolabled anti-breast tumor antibodies and other radiolabeled agents have met with similar mixed results. Accordingly there is a need for improved appraoches for the detection and treatment of breast tumors. This work explored several novel approaches using DNA for specific targeting and signal amplification. The work completed during this period will serve as the foundation fur future work in this area.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
17

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

FOREWORD

Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

____ Where copyrighted material is quoted, permission has been obtained to use such material.

____ Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

____ Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

____ In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

✓ For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

✓ In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

✓ In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

____ In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

CE Snell   11/1/99

PI - Signature   Date

5. INTRODUCTION

Considerable interest has been generated in the use of nucleic acids for therapeutic use (1-3). Exciting examples of anti-sense applicatons include slective inhibition of viral RNA replication or gene expression (4-6). Other in vivo applicatons have included oligonucletocide-antibody conjugates for tumor diagnosis and therapy (7).

This interested promoted the investigation in model systems of the pharmacokinetics, biodistribtuions and stablity of olgionucleotdies in vivo ranging in size from 12 bases (8) to as large as 38 bases (9) with radiolabels, such as ^{125}I (7,10,11), ^{32}P (9/12, ^{35}S (12, 13, 14) and ^{111}In (15, 16). In these and other in vivo applciations the nucleotide backbone was modified to prevent nuclease degration, typically with methylphosphonates (17-20) or phosphorothioates (21-24), although terminal modification alone was shown to provide stability (9, 13, 20, 25).

Amplification techniques for nucleic acids are under active investigation for in vitro applicatons. These iclude PCR (26), the "christmas tree" approach (27), and brancing nucleic acid dendrimers (28-34). However, using nucleic acids for amplification in vivo has not previously been considered.

The overall goal of this project is to form at the site of a tumor a large aggregate. The complex is formed, de novo, at that site of tumor from small molecules which self-aggregate. The formation of the aggregate means that the original tumor target signal is amplified. Then, the aggregate at the stie of the tumor is used as the target of a small molecule carrying a low molecular weight label such as an imageable readionuclide. This proposal describes the assembly in situ of an aggregate possessing what could be an almost limitless amount of radioactivity while at hte same time the method seeks to minimize radioactivity in normal tissue. Thus, the tumor tissue radioactivity ratios should be exceptionally high for both diagnostic and therapeutic applcations.

This approach is based upon several well characerized high affinity

systems (double stranded DNA, streptavidin-biotin, and leucine zipper dimerization) that are being combined in a unique fashion. Nucleic acids and their derivatives were chosen becaue (1) the affinity and specificity between two complementary DNA sequences in natural settings is quite efficient and tight, (2) short DNA molecules shold be non-toxic and (3) DNA molecules can be radiolabeled relatively easity via a number of strategies. A streptavidin-biotin system was chosen because of (1) the high stability of this protein in vitro and in vivo and its low immunogenicity, (2) the existence and ongoing construction, of a variety of potentially useful recombinant streptavidins, (33), the ease of biotinylation of a wide variety of molecules. The dimerizing leucine zipper recmobinant constructs was choosen becasue of (1) the specificity and stability of the dimer and the fact that the zipper can be formed with a short oligopeptide The three novel amplification strategies to be tested here are (1) a protein-nucleic acid aggregate consisting of a pair of streptavidin conjugated with complementary single stranded DNAs (or PNAs), (b) a pure protein aggregate consisting of pairs of streptavidin fused to the ends of the leuzine zipper. One zipper is fused to the N-terminus of streptavidin and the other is fused to the c-terminus. It is also anticipated tha the nucleci acid amplification schemes described herein will have applictions which extend far beyond this proposal.

The formation of aggregates at tumor sites can be broken down into three stages (1) initialization, and (2) amplification and (3) termination. Although the primary focus on this research was on the amplification stage, exciting results on other experiments focused on the initializing step lead up to concentrate our efforts in that area.

The initializing or targeting of reagents to tumor cells critical to this and other efforts to treat cancer. In fact, a major problem in medicine is delivering a therapeutic agent to a target organ. Currently, most medication is not targeted but rather distributed throughout the body. This means that therapeutic levels are schieved after excess amounts of medication is given to a patient. Drug doese are chosen that provide an effective level to the target cells and minimize damage to bystander tissues. A recentt elegant solution being expolored by some researcher is to place medication on magnetic beads and then target the appropriate organ using an extenal magnet. This may be appropriate for large organs

like the heart, etc. However, this approach is not likey to work with solid tumors because of multiple micrometatases at secondary sites being a primary factor morbidity.

Antibody targeting of tumor cells does not work well. In general, tumor specific antibodies have been difficult or impossible to develop. The problem initilization was in fact highlighted as the major criticism of the original proposal. Antibodies tend to get stuck int he kidneys and have problems penetrating solid tumors. Hence, experiments, supported by other funds were used to explore the use of nucleic acids for tumor targeting. The goal of those experiments was to isolated a DNA mimics of antibodies, called aptamers (meaning in Greek, "to fit") specific for tumor cells. The advantage of aptamers is their small size and the ability to select specific, high affinity apaters from large libraries of random nucleic acid sequences. Nucleic acids are non-immunogenic. When other funds, supporting that work ran out, the aptamer project was continued with funds from this project. Progress on initilization step (aptamer project) and amplification step will be discussed below.

6. BODY

Materials: Oligonucleotides used in this work were purchased from Operon Technologies (Alameda, Ca). Where needed the nucleic acid swill be flourescently labeled for direct imaging of aggregates with flourescent digital miscroscope or end-labeled by us with ^{32}P using conventional DNA kinasing methods. Biotinylated oligonucleotides were purchased as needed. Streptavidin coated microbeads were purchased from Dynal Inc. (Oslo, Norway). Tumor cell lines were purchased from the American Type Culture Collection (ATCC). DNA sequence analysis was done using GCG software provided by the BMERC facility of Boston University and by freeware MEME and MAST. DNA sequencing kits were purchased from Pharmacia. Carcinogenic embryonic antigen (CEA), concanavalan A and alpha-mannose was purchased from Sigma. Chemically reactive biotin was purchased from Aldrich. DNA cloning and sequencing was done using commericial kits from Clontech and Pharmacia, Inc. The automatic flouresent ALF-express sequencer was used for sequence determinations.

Methods: (A) Aggregate Formation: Several very simple systems were

set up to explore aggregate formation in solution and on surfaces. A general schematic of the approach for in vivo targeting is illustrated in Figure 1. A first step (initializing step) would involve the administrative of a tumor-specific molecule tagged with a linker to the aggregation system. This could be an antibody directly conjugated with single stranded DNA molecules or an anti-tumor aptamers with a single strated tail. This is followed by the administration of a streptavidin conjugate (or pure DNA species in the form of a Holliday structure with four single stranded ends). Potentially there are found sites on tetrameric streptavidin for biotinylated oligonucleotides to binds. The single stranded regions should be at least 20 to 24 bases in length and one of the single stranded oligonucleotides/streptavidin molecule compleentary to the single stranded species on the initilizer. The third step in the procedure is the addition of a secon streptavidin species with oligonucleotides complementary to the the remaining single stranded ends. Althernating injections of complementary oligonucleodies should produce a large aggregate at the site of a tumor. The final step would be the addtiion of an radiolabeled single stranded DNA, complementary to most recently injected oligonucleotide.

Potentially, each cycle of addition of complementary DNAs strands for tetra-valent reagents, will results in an upto three-fold increase int he number of strands added to the aggregate. This amplification is potentially greater than that of PCR which makes it an attractive candidate for in in vitro applications as well. Using these methods it should be possible to construct an aggregate at each antigenic site which containins an exponential increase int he amount of radiolabeled achievable by direct labeling experiments. Moreover, the radioactivity should be concentrted at the tumor due to the quick cleqrnace of the low molecular weight radiolabled strands from circulation and normal tissues.

(B) Anti-CEA Aptamers: CEA was choosen as a first aptamer target for tumor targeting because of the enormous amount of work over the last 40 years using anti-CEA antibodies. Although anti-CEA antibodies are effective targeting reagenets, the large number of anti-CEA antibody expeirments can serve as standards for establishing the behavior of anti-CEA aptamers. Potential anti-CEA aptamers were selected from random DNA oligonucleotide libraries with a complexity of $\sim 10^{15}$ using methods

developed by us (Smith et al., unpublished results). A DNA library for aptamers was p repared from a100-base length single stranded DNA. The DNA oligonucleotides has a 64-base variable sequence flanked by two 18-base constant sequences. The constant sequences are used as primer-binding sites for PCR amplification. These experiments were begun using a conventional aptamer selection appraoch. Conventional aptamer selection methods incubate an immobilized target molecules, in this case CEA, with an oligonucleotide library of random sequences. This set up allows unbound oligonucleotides to be removed efficiently. The conventional aptamer selection protocol was used without success. It then became apparent that the failure was because the selection protocol was diffusion limited by the low concentration of CEA and further by its immobilization. This meant that CEA would not interrogate a large portion of the library unless extensive (weeks) incubation times were used. Hence, the procedure was modified so that CEA was incubated with the oligonucleotide library in solution. After sufficient time, the target plus bound oligonucletoides were immobilized and the unbound oligonucleotide removed. Using the modified selection protocol, a library of potential anti-CEA aptamers was created. The success of the selection protocol is likely because (1) about 20% of the selected library bound to CEA after 7 rounds of selection when <1 % bound initially and (2) specific restriction fragments could be detected in the oligonucleotide pool after 4 rounds of selection, while none were detected in the first three selection rounds. In this work, the library from after the seventh round of selection was cloned and sequenced using conventional methods. The sequences were analyzed using several multiple alignment program to identify shared motifs.

Results: (A) Aggregate Formation: Two model sytems were set up to study layer formation in vitro. In addition, studies on a simple tumor model system was also begun. One in vitro system followed aggreagate formation in solution and the other followed aggregate formation on a surface. The solution system used stretavidin coated beads. The beads were divided into two batches. Biotinylated complementary DNAs were used to link the beads together (Figure 2). On aveage, the aggregations were composed of 4 beads in diameter. In the surface aggregation system, flourescently labeled streptavidin was used to follow the formation of the aggregation. One can envision such a surface as the outside layer of a

tumor. The results of such an experiment are shown in Figure 3. The quantitative data shown that a 50-mer linker allowed four layers to be build up. AT this time it is not clear why the aggregate size could not be expented beyond four layers but there are many vaiables to be tested. However, it may be that the geometry of the biotinylated DNA stranded emerging fromt he bioting binding sites of streptavidin sterically hinder the construction of more layers. This means that the formation of aggregates might best be done using pure DNA molecules.

The model system developed with tumor cells involved the immobilization of single stranded DNA on the surface of a tumor cell. This oligonucleotide served as the initializing target to study the formation of an aggregates on tumor cells (Figure 4). The protocol involved developing a protocol for biotinylating the surface of a whole tumor cells without affecting viability using standard chemical methods. Then, adding streptavidin with biotinylated oligonucleodies. After this aggregate formation would proceed as outlined above. The experimental results demonstracted that tumor targeting in this model sytem could be accomplished with DNA and that the DNA was stable on the cell surface for at least 24-hours.

(B) Anti-CEA aptamers: About 100 individual oligonucleotides were cloned and sequenced using conventional methods after 10 rounds of selection when about 20% of the library bound to the target (Table 1). After four rounds of selection, specific restriction fragment cleavage sitescould be detected in the the selected pools (Figure 5) indicating that the complexity of the pool was greatly reduced. Several multiple alignment programs were used to compare the sequences and develop a minimum unique sequence group (Table 2). Several software programs including PILEUP in GCG and MEME and MAST were used to search for conserved sequence motifs in the minimal set. Three motifs were identified. The affinity of these sequences for CEA are now being measured.

7. KEY RESEARCH ACCOMPLISHMENTS

The goal of this research is to use DNA as therapeutics for cancer tumor cell treatment. The research program explored a number of aspects or this approach and has produced a foundation on which additional research can be done. Here, the initialization and amplification steps were explored. The most significant accomplishment was the isolation of potential anti-CEA aptamers. The other accomplishments include the set up of model systems using tumors cells and beads.

8. REPORTABLE OUTCOMES

Grant Applications:

Cassandra L. Smith (PI), Tumor Targeting with DNA Aptamers, DOE, 1998 - not funded

Cassandra L. Smith (PI), Anti-Tumor Targeting, Boston University Provost Innovation Fund, 1999 - funded $25,000

Gregg Surdi, Pre-doctoral Fellowship Application. Targeting Breast Cancer with Anti-CEA, DOA Breast Cancer Program, 1999 - pending

Presentations:

Cassandra L. Smith, 1999. Physics Colloquium at Boston University, "Macromolecular DNA Structures"

Patent Application:

Disclosures about this technology have been given to the Boston University Patent Office who is decided on whether to file patents.

Publications:

It is anticipated that there will be at the minimum one publication from

this work once the work is completed, although it is more likely to be multiple publications from this work.

9. CONCLUSIONS

The funds provided by the Department of Army enticed the principle investigator to begin thinking about breast cancer disease and to develop a completely new line of research based on DNA therapeutics. As with truly new research directions, progress has been slower than predicted, but promising enough so that this work will continue even in the presence of very limited funds.

The principle investigator believes that the demonstration of the specificity of the aptamers is key for demonstrating the feasability of the using DNA therapeutics for breast cancer disease treatment. Hence, the current focus of our research is on confirming the specificity of the aptamers that have been isolated to provide the initializing step

The amplification (aggregate) step needs to be further developed theoretically and in vitro as there is little published research in the area of homogenous and heterogenous molecular structures in vitro. Some of the most promising work has been reported by Seeman, 1995, 1998. It is quite clear that nucleic acids are versatile tools for building molecular structures for a variety of purpose because they allow fast prototyping and virtually any sequence can be made rapidly and easily. Hence, it is anticipated that this work will continue once more funds are available.

10. REFERENCES:

TL Bailey and CL Elkan. 1994. Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology. pp 28-36 AAAI press, Menlo Par, California.

T Bailey and M Gribskov. 1998. Bioinformatics 14: 48.

G Carter, NR Lemoine. 1993. Br. J. Cancer 67: 869.

M Kulka, M Wachsman, S Miura, R Fishelevich, PS Miller, POP Tso, L Aurelian. 1991. Antivial Res. 20:155.

CK Mirabelli, CF Bennett, K Anderson, ST Crooke. 1991. Anti-Cancer Drug Design 6: 647.

AD Ellington and JW Szostak. 1992. Nature 355: 859.

NC Seeman 1995. The Chemical Intelligencer, pp. 38.

NC Seeman. 1998. Ann. Rev. Biophys. Biomol. Structure, 27: 225.

CA Stein, and JS Cohen. 1988. Cancer Res. 48: 2659.

CA Stein, YC Cheng. 1993. Science 261: 1004.

E Uhlamnn, Peyman. 1990. Chemical Rev. 90:543-584.

Wisconsin Pakcage Version 9.1, Genetics Computing Group (GCG), Madison, Wis.

Tables 1 Binding of oligonucleotide libraries to CEA after each round of selection. The percent bound was determined by measuring the amount of 32P-labled oligonucleotide immobilized with CEA.

Selection Rounds	CEA µg(µM)	DNA µg(µM)	Unbound (ml)	Bound (ml)	% Bound
1	200 (~2)	76(~4)	5.7	7.2	5.99
2	200	27	6.5	6.4	0.91
3	200	6.4	7.0	7.0	0.42
4	200	10	7.3	7.2	0.61
5	200	9	17	7.5	2.93
6	200	6.7	19.5	7.5	18.9
7	-	3.3	-	-	-
8	-	3.3	-	-	-
9	200	2.9	20	7.0	9.9
10	200	3.2	28	7	21.1

Table 2. Potential anti-CEA aptamers. Afther the tenth round of selection, oligonucleotides were clones and sequenced. A minimum unique set of aptamers were aligned and three recurring motif were identified.

```
clone45  ---------- --GGTGGGGG GATTCCCTCG TTTATTTACG TYTCTGAYAT GGTTcCGCTC ACGCTCCCCC T
clone48  ~~~~~GGATG GTAGTCGGGG TATCCTTTTA AGGAGTTAGT CAACGAGCGT TAGTATTTTA ATGTTCCC~~ -
clone60  ~~~AGACGAG GBMAGTGGGG sAGTACCY.V CTV.GVAGTT TAADGSSB.K BCBTAT.ATT CCACGGBTGA T
clone66  ---AGACGA. GGAAGTGGGG GAGTACCC.G CTG.GGAGTT CAAAGCAT.T TCTTAT.ATT CCACGGCTGA T
clone52  ~~~AGACGA. GSAAGTGGCG GAGTACCC.G CTG.GGAGTT CAAAGGGBGB SCBTAT.ATT CCTCGCCCC~ -
clone72  ~~~AGACGA. GGAAGTSGGG GAGTACCC.G CTG.GGAGTT CAAAGCAT.T TCTTAT.ATT SCTCGCCCC- -
clone16  ---GGVAGAV GG.TGAGGSG GCGTACCC.G CCGTGGAGGA TATCGGTATT AGACATCAGT TCTTTTCCG- ~
clone22  ~~~GGGGGAG GGGaCGACGC GATACCCC.G CTTAGAT.TA TACGTTTGGT ATTCATTTCT GCCGCCCTT~ ~
clone39  ~~GGGGGAAG GGGGCGACGC GATACCCCGG CTTAGAT.TA TACGTTTGGT ATTCATTTCT GCCGCCCTT~ ~
clone75  ---GGGGTAG GGGSCGACGC GATACCCS.G CBBVGVT.TA TACGTTTGGT ATTCATTTCT GCCGCCCTT- ~
clone23  ~~AGGGGAAG GGGCTGAAAG GATACCCCTG TTGATCTGTG TAACACCGATT AGTTATATCT ACTTCC---- ~
clone74  ---GGGGTAG GGGGCGAAGC GATACCCCTG NTGATCTGTG TAACVCSVSS VGBTATATCT ACTTCC---- -
clone26  ---GGGGTAG GGGaCGAAGC GATA.CCCTA ATCAGCGTCC TA..GCGGCT TCCGGTTTCG TCTTTTCG-- ~
clone11  ~~~~GGCGAG GGGAAGGGAG GAGTCCTAAG GGGTACTGTA GGGAGYTRGT TTTTTAACCT cCCTTT---- -
clone78  ~~~~GGGGGG GTAGGGGGCC TAGCCCTCTT CATTAATK.. GGRAGCCGCG TTTTTCTCGA G--------- -
clone41  ---------- ACCATGAGGG GGGGCGTAAG CCTACAAGCA TACGCATGGC TATTCTTTCA GCGTCTCGTA ~
clone57  -GCAGGAGTG TGAAGGAATT GATGG.GGgC GGGGGGTGAA GCGACACSCC TTTCAACTCT TCGTCG---- ~
clone62  ~~~~~GGGAG GGTGGG.ACG GTTGC.GGTG GTTGGGGTAA GGCCCACCSC CTTTAGC.CT TCGVTTTCCC T
clone9   AGAAGGGGGG ACCAGGGCTA GTGACTGGgC ATGAAGTGAA GGACGGTACA TTCCAATTGG CTGT------ ~
clone51  ~~~~ATGATA GAGG.TGGGT GCGCTCCTTG A.GGTGGGGA GCGGGACGAG TGTGACACCC CGGTTG---- -
clone71  ~ATCGGGATT GTSGTTCCGA GATGCCATTG ATAGTGAYGA TTTYNNVCRG .GTGAACCCG CTGTGG---- -
clone76  ~~~~GGNGGC TTGGTCCGGT GAATTCTGTA AGAGTTTTAT TCTAGACTCG CTTGTTTCCT TGCCAG---- ~
```

```
Motif 1              Motif 2          Motif 3
AGGGGGTGAAGGGATACCC  TATTTTTTTCG      CTGCTGATCTGTGTAA
G  AATC GGACAG T       GACACCA T       C TGTTAT C CG G
     A      C              A            G
```

Figure 1. Schematic of aggregates fromed using STreptavidin-Streptavidin-biotinlylated linkers. Circles represent tetrameric streptavidin. Bis-biotinylated double stranded straded DNA serves as linker between streptavidin molecules.

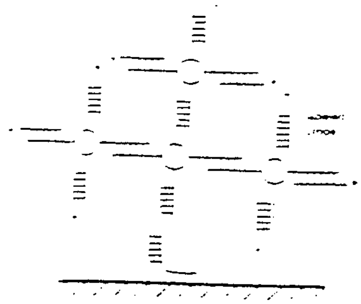

Figure 2. Formation of Strepativind coated microbead-biotinlylated DNA aggregates in solution. (A) aggregates of streptavidin coated microbeads through a bis-biotinylated double stranded DNA. (B) Beads with non-complementary DNAs do not aggregate.

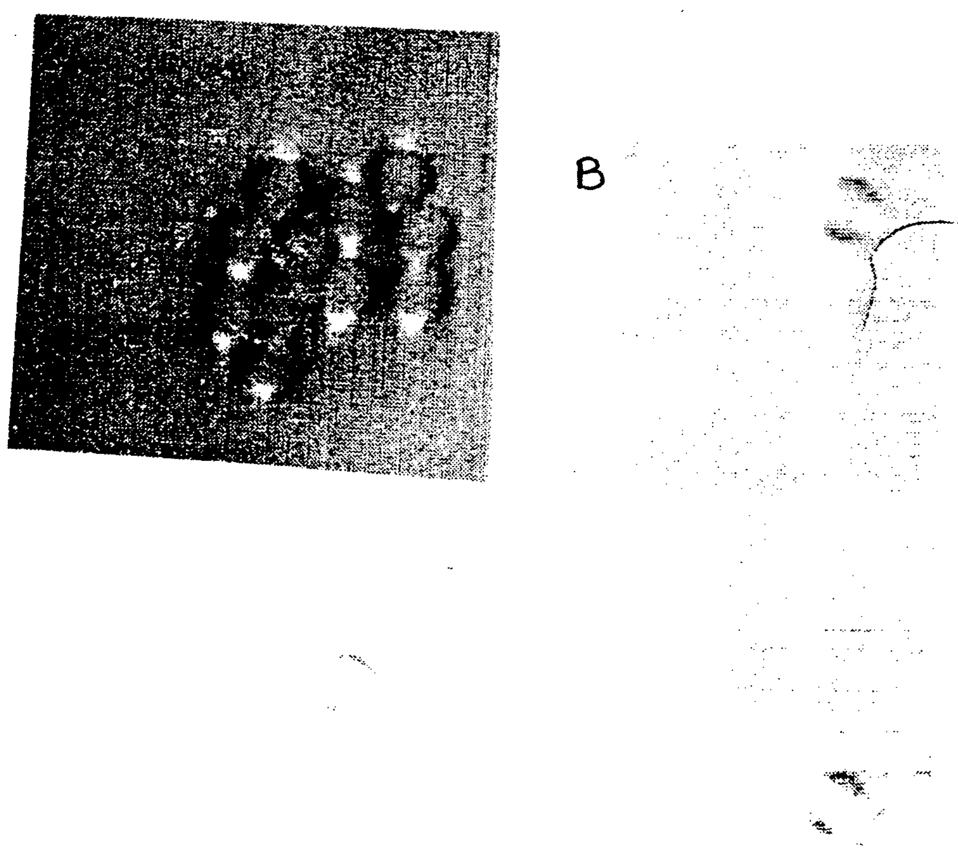

Figure 3. Formation of Streptavidin-biotinylated DNA aggregates ona silicon surface (see text for details)

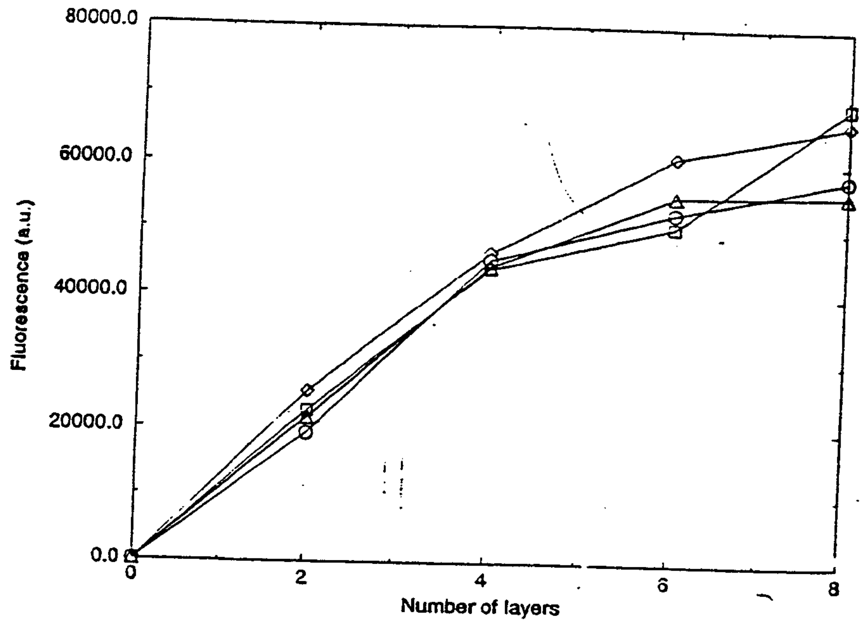

Figure 4. Target single stranded DNA immoblized on the surface of tumor cells. Breast cancer tumor cells grown in tissue culture were chemical methods with doses that did not affect viability. The cells were incubated with streptavidin followedby incubation with biotinylated DNA then followed by incubation with complementary (A) or non-complementary (B) 32P-labeled DNA.

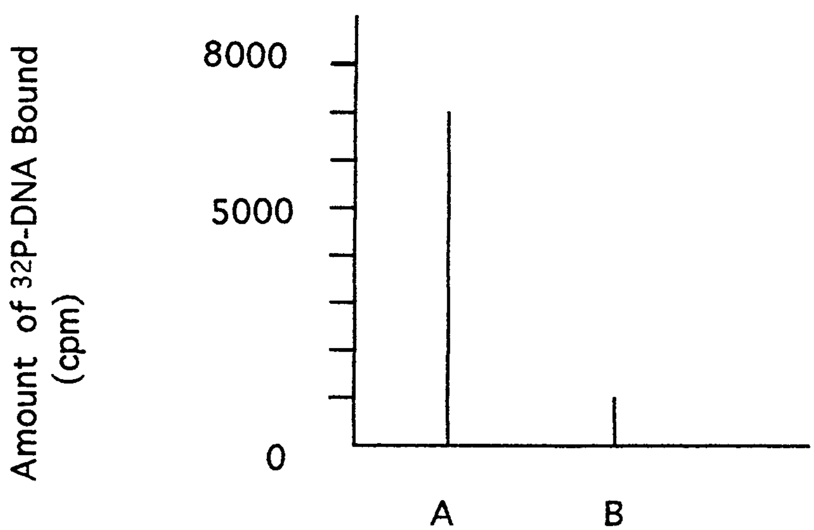

Figure 5. Oligonucleodite library Complexity. After each selection cycles a fraction of the DNA was re-amplified by PCR with one ^{32}P-labeled primer. The 100 base PCR produced were gel purified using a Qiagen kit and digested with three restriction endonucleases (Sau3A I, Hinf I and Aci I) and fractioned by size on a denaturing 8% polyacrylaminde gel containing 7 M urea. The occurrence of specific bands is indicative of low complexity samples.